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Investigation of chitosan-glycol/glyoxal as an injectable biomaterial for vocal fold tissue engineering

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Abstract

Injectable hydrogels offer promising tissue engineering approaches for vocal fold (VF) tissue repair. Research on VF tissue engineering scaffolds has largely been focused on derivatives of hyaluronan and collagen. Although chitosan hydrogels have been extensively investigated for various soft tissues, their potential use for VF tissue engineering has been overlooked. The aim of the present study was to investigate cross-linked Chitosan-glycol (GCs)/glyoxal (Gy) hydrogels for VFLP tissue engineering. The effects of Gy concentration on cell viability, viscoelastic properties, enzymatic degradation, and cell migration were studied. Six different groups of cell-seeded hydrogels, consisting of immortalized human vocal fold fibroblasts encapsulated in GCs/Gy hydrogels, were prepared to obtain target concentrations of 2×10^6 cells/ml, GCs 2% and Gy 0.02% (Group#1), 0.015% (Group#2), 0.01% (Group#3), 0.0075% (Group#4), 0.005% (Group#5), or 0.0025% (Group#6). The storage and loss moduli were 629±35Pa and 9±1 Pa, 560±28 Pa and 9±1Pa, 489±41 Pa and 8±1 Pa, 307±25 Pa and 4±1 Pa, 149±31 Pa and 3±1 Pa, 55±17 Pa and 3±1 Pa for groups 1, 2, 3, 4, 5, and 6, respectively. The viability rates were above 90% for all groups, 3 hours after encapsulation. The viability rates were 60.0±2.2%, 80.3±2.2%, 83.5±0.5%, 83.1±1.3%, 88.2±0.1%, and 88.0±1.1% for groups 1,2,3,4,5, and 6, respectively, one week after encapsulation inside GCs/Gy hydrogels. The average cell motility speed was 0.09±0.03 µm/minute, 0.07±0.043 µm/minute, and 0.09±0.02 µm/minute for groups 4, 5, and 6, respectively. Following four weeks enzymatic degradation study, the mass loss was 10%, 21%, and 100% for groups 4, 5, and 6, respectively. Our results indicated that GCs/Gy hydrogels could be potential candidates for use in human VF tissue repair and regeneration.

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1. Introduction

The human vocal folds (VFs), located within the larynx, include soft connective tissues, such as the epithelium, the basement membrane zone, and the lamina propria (LP). These tissues are attached to the vocalis muscle and anchored to the thyroid and arytenoid cartilages [1]. In normal phonation, the VF mucosa and ligament undergo vibrations at frequencies ranging from 20 Hz to 3 kHz and amplitudes of a few millimeters. Voice disorders can impose occupational risks especially for teachers, signers, and public speakers, and may also have emotional consequences.

Scarring is a common voice disorder. Vocal fold scarring affects the mucosa and the LP. It may result from the surgical removal of benign or malignant VF lesions, phonotrauma or intubation over an extended period of time. Early scarring appears one to three months following VF injury, and may take up to twelve months for the remodeling phase completion and scarred VFLP maturation. Scarred VF tissue is fibrotic with diminished elastin but excessive disorganized collagen deposition [2]. As a result, the viscoelastic properties of the LP are significantly altered. Using a rabbit model, it was found that the stiffness and dynamic viscosity of the LP are one order of magnitude larger in scarred LP than in normal tissue [3]. Since the mechanical properties of the LP must be within a specific range to ensure proper phonation, viscoelastic biomaterials are often injected to treat scarring by compensating for the excessive stiffness of the scarred tissue. But, these materials degrade over time, and periodic re-injection is required. This shortcoming may be avoided through the use of tissue engineering approaches, which are intended to effectively modulate wound healing and regenerate functional VF tissue. The main motivation for the current investigation is to design, fabricate, characterize, and examine a new injectable biomaterial for VF tissue engineering.

Injectable hydrogels offer promising tissue engineering approaches for VF tissue healing. Biomaterials based on natural hydrogels such as hyaluronic acid (HA), gelatin (Ge), chitosan, alginate and fibrin have been used to promote soft tissue regeneration [4-6]. Vocal fold tissue engineering investigations have been largely focused on derivatives of hyaluronic acid and collagen. These macromolecules are the main components of the VFLP extracellular matrix. Hyaluronic acid is a non-sulfated glycosaminoglycan and a major contributor to maintain VF tissue viscosity. The HA-Ge hydrogels cross-linked by disulfide bond formation are biocompatible [7] and has viscoelastic properties similar to VFLP [8]. The injection of HA-Ge hydrogels in rabbits after unilateral injury resulted in less fibrotic tissue. Favorable biomechanical properties were achieved compared to those of the controls injected with saline. One major problem with bulk HA-based hydrogels is their fast degradation *in vivo*. Alternatively, an injectable biomaterial based on densely cross-linked micro-gels of HA and Ge was developed [9], and was shown to be biocompatible in a preliminary animal study [10].

Chitosan is a linear poly-saccharide containing heteropolymer of randomly distributed β-(1,4)-linked D-glucosamine and N-acetyl-D-glucosamine units [11]. Chitosan, derived from chitin by partial deacetylation, is the second most abundant natural biopolymer [12]. Similarities to glycosaminoglycans, abundance in nature, biocompatibility, low production cost, and low immune-stimulatory activities have made this polymer very appealing for drug delivery, wound healing, and tissue engineering applications [13]. Chitosan is biodegradable, and can be metabolized by certain human enzymes, especially lysozyme. Chitosan hydrogels have extensively been investigated for variety of soft tissues such as skin [14], cartilage [15], blood vessels [16], and brain [17]. However, they have not been investigated for their potential use in VF tissue engineering.

Ionic and covalent cross-linking are the most common methods for the fabrication of chitosan hydrogels. Covalent cross-linking forms a permanent network, and therefore the hydrogels may reside longer *in vivo*, thereby better for tissue engineering applications. Ionic cross-linking, in contrast, is more prone to break down with the body fluids, and is then more appropriate for drug delivery applications [18].

The aim of the present study was to investigate cross-linked Glycol Chitosan (GCs)/glyoxal (Gy) hydrogels for VFLP tissue engineering applications. The effects of cross-linker (Gy) concentration on cell viability, viscoelastic properties, enzymatic degradation, and cell migration kinematics were studied.

2. Materials and Methods

2.1. Cell Culture in Flask

Immortalized human VF fibroblasts (I-HVFFs) were cultured in a mixture of Dulbecco's Modified Eagle Medium (Life Technologies Inc., Burlington, ON), 10% fetal bovine serum (Sigma-Aldrich Corporate, St. Louis, MO), 1% Penicillin/Streptomycin (Sigma-Aldrich Corporate), and 1% MEM non-essential amino acids (Sigma-Aldrich Corporate) at 37°C, in 5% CO_2 humidified atmosphere. The cell culture medium (CCM) was replaced every three days. Cells were disassociated using 0.25% trypsin-EDTA when the cell confluency reached 70%.

2.2. Cell-Seeded Hydrogel Preparation

Glycol-Chitosan and Glyoxal 40% were purchased from Sigma Aldrich Corporate. Solutions of GCs 4% and Gy 10% in deionized water were prepared, and placed in a rugged laboratory rotator (Glas-Col Corporate, Terre Haute, IN) at 30 rpm for 24 consecutive hours. The solutions were subsequently autoclaved to avoid contamination. Six different groups of cell-seeded hydrogels were prepared to obtain target concentrations of 2×10^6 cells/ml, GCs 2%, and Gy 0.02% (Group#1), 0.015% (Group#2), 0.01% (Group#3), 0.0075% (Group#4), 0.005% (Group#5), or 0.0025% (Group#6).

2.3. Rheometry

A TA Instrument Rheometer-AR2000 (New Castle, DE) was used to measure the shear elastic and loss moduli of the hydrogels at room temperature. Parallel plates with a diameter of 20 mm and a gap of 200 μm were used. The samples were prepared and molded to be cylindrical in shape, nominally 20 mm in diameter and 1.5 mm in thickness. The samples were sealed and kept at 37°C, in humidified atmosphere for 24 hours. They were then properly placed to fill the gap between the plates. They were surrounded by water in order to prevent dehydration. A controlled stress frequency sweep test was performed for the frequency range between 0.5 Hz and 5 Hz.

2.4. Enzymatic Degradation

The enzymatic degradation of the hydrogels was investigated using lysozyme in PBS 1X for hydrogels with the three lower concentrations of Glyoxal (0.0075% (Group#4), 0.005% (Group#5), or 0.0025% (Group#6)). A volume of 0.5 ml GCs-Gy was poured in each vial. PBS 1X (1 mL) was added into the vials two hours after preparation. The vials were then placed inside a humidified atmosphere at 37ºC, and gently agitated at 75 rpm. The medium was changed every eight hours. After 24 hours, the medium was removed, and the hydrogel weight was measured. The hydrogels were then incubated in the solution of 13.0 μg/ml lysozyme in PBS 1X, with gentle mechanical agitation at 75 rpm over the period of study. This lysozyme concentration corresponds to that in human serum [19] . The solution was refreshed daily to ensure continuous enzyme activity. After three days, one week, two weeks, and one month, the medium was removed carefully, the samples were lyophilized, and the dry weights of the hydrogels were measured. The weight of the dried hydrogels was calculated by subtraction of the empty vial weight from the measured data. The remaining weight fraction, i.e., $W\tau/W_0$ in which W_0 is the original dry weight of the hydrogels, and $W\tau$ is the associated dry weight at time τ, was computed and plotted with respect to time. The extent of *in vitro* degradation was expressed as the weight fraction remaining of the dried hydrogels at the specific time intervals, i.e., $W\tau/W_0$.

2.5. Cell Viability

The LIVE/DEAD® Viability/Cytotoxicity kit (Life Technologies Inc., Burlington, ON) was used to assess the viability of cells in the hydrogel samples at the following time points: three hours, three days, and a week after preparation. The kit provides a two-color fluorescent image. Calcein-AM and Ethidium homodimer-1 (EthD-1)

specifically stain live and dead cells, respectively. The samples were washed for 3×5 minutes (i.e., three times, each for 5 minutes) using 1X Phosphate-buffered saline (PBS). They were then incubated with working solution (2 μM calcein-AM and 4 μM EthD-1 in DPBS (Life Technologies Inc., Burlington, ON)) for 30 minutes in darkness at room temperature. The samples were washed again in 1X PBS for 3×5 minutes. An inverted confocal fluorescence microscope (LSM710, Zeiss, Jena, Germany) was used to image the stained samples. All the images were acquired using a 10X objective (10x/0.45 Dry Plan-Apochromat, Zeiss). Series of XY images, called Z-stack, were obtained. Image acquisition and image analysis were performed using the software Zen (Zeiss) and Imaris version 7.5.6 (Bitplane, South Windsor, CT), respectively. The viability rate was then obtained by dividing the number of the live cells to the total number of cells (the number of both live and dead cells) in each reconstructed image. The normalized number of cells was then obtained by dividing the number of cells (live and dead) with the volume of the reconstructed image.

2.6. Migration

Vybrant® DiD Cell-Labeling Solution (Life Technologies Inc.) was used to stain the cell membrane. The cells were disassociated using 0.25% trypsin-EDTA. They were subsequently centrifuged and suspended in serum-free CCM. 5μl of the labelling solution was added to each 1×10^6 cells. The cell solution was then incubated at 37°C for 20 minutes. It was then centrifuged for 5 min at 1.5×10^3 rpm. The solution was then replaced with serum-free CCM, and pipetted up and down to mix thoroughly. The solution was again centrifuged, and replaced with serum-free CCM. The labelled cells were used to prepare cell-mixture solutions to obtain target concentrations of 5×10^5 cells/ml, GCs 2%, and Gy 0.0075% (Group#4), 0.005% (Group#5), or 0.0025% (Group#6). An inverted confocal fluorescence microscope (LSM710, Zeiss) was used to image each sample for four consecutive hours. Image analysis was performed using Imaris version 7.5.6 (Bitplane).

3. Results and Discussion

3.1. Cell Viability

Representative images of viability tests for group#2, group#4, and group#6 are shown in Fig. 1. The results indicated that the high concentration of Glyoxal cross-linker can cause cytotoxicity to human fibroblast cells, as shown in Fig. 2. Glyoxal was not cytotoxic up to three hours after mixing with the cells for any of the groups under investigation. However, group #1 and to some extent group #2 showed significant cytotoxicity after three days. Glyoxal is a reactive α-oxoaldehyde and works as glycating agent in all tissues and body fluids in physiological conditions. Glyoxal is capable of inducing significant cellular damage above 5 mM (0.025%) by oxidative stress [20]. All of the studied groups had concentration below 0.025%. Not surprisingly, the group#1 with glyoxal concentration of 0.02% had still exhibited toxicity after three days. The concentration of glyoxal under physiological condition is around 12.5 μg/ml (0.00125%) [21], which is lower than that of the groups studied. It should be noted that glyoxal cross-linking with chitosan starts immediately after blending the ingredients and the cell solution. This lowers the concentration of free glyoxal in the mixture, and mitigates its cytotoxic effects. Furthermore, glyoxal is detoxified enzymatically by cytosolic glyoxalase system [20].

3.2. Rheology

Shear storage and loss moduli of the fabricated hydrogels are shown in table 1. An increase in the Gy concentration yielded greater storage and loss moduli. Fine-tuned viscoelastic properties of injectable biomaterials are essential to restore the functionality of differentiated VF tissue. The VFLP undergoes complex mechanical loading during phonation, which influences the structural and functional properties of the VF tissue during development [22]. In a study using a phono-mimetic bioreactor, it was shown that the mechanical properties of the injectable biomaterials have a significant impact on onset phonatory characteristics [23].

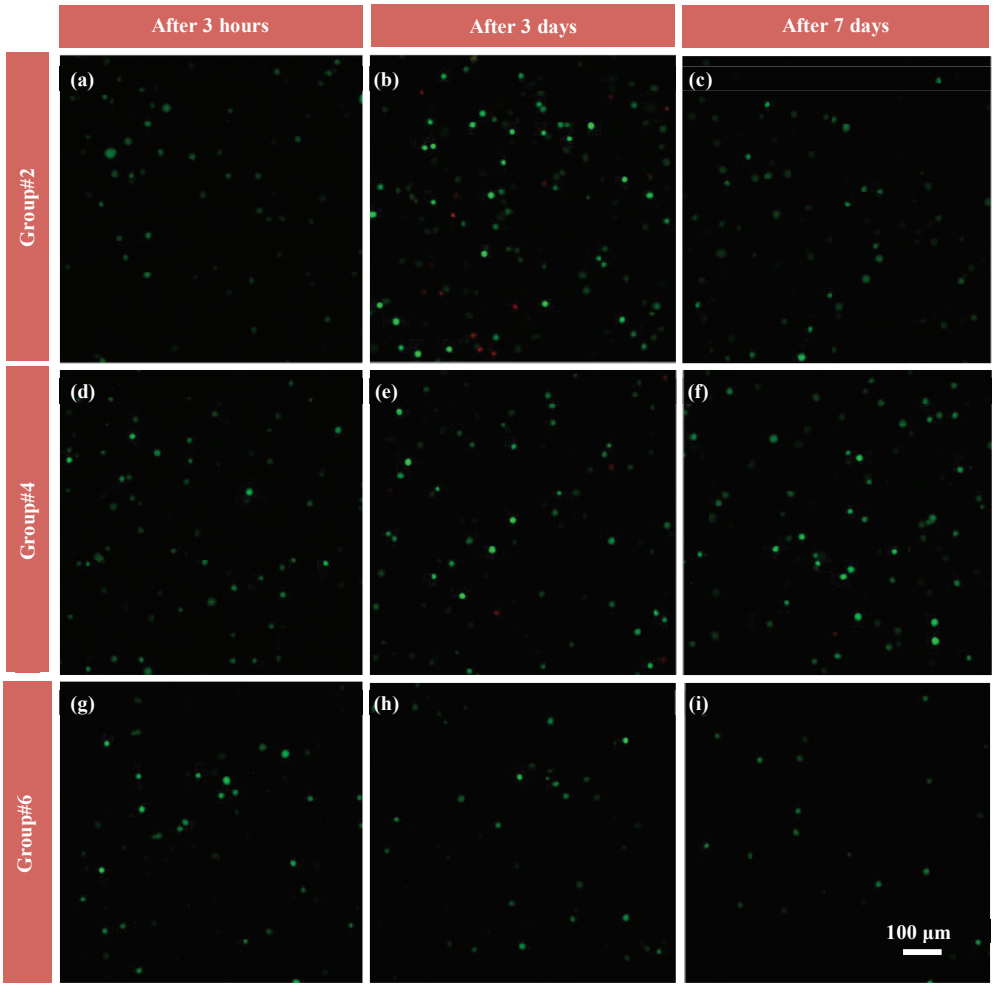

Fig. 1. Fluorescence images of I-HVFFs in the Cs-Glayoxal hydrogel. Viable cells are green-fluorescent and dead cells are red-fluorescent, respectively.

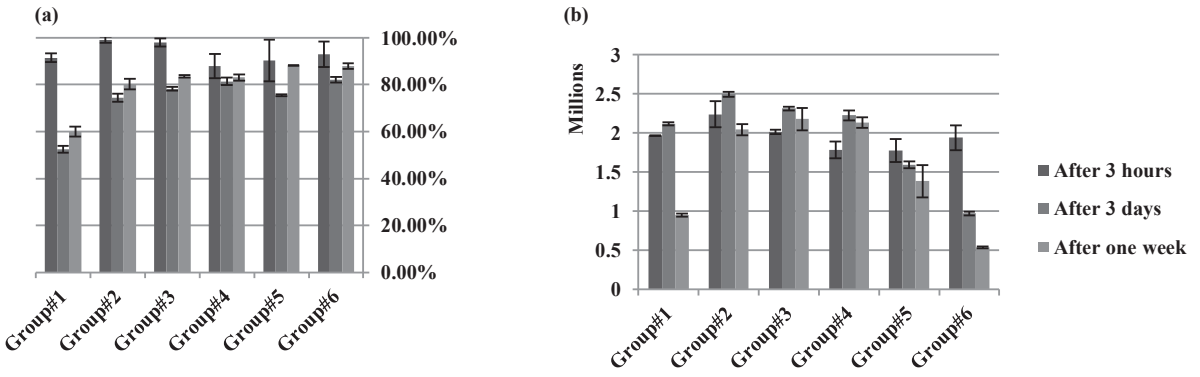

Fig. 2. (a) Viability rates of six chitosan/glyoxal hydrogel groups with different cross-linking concentrations. (b) Normalized total number of cells per volume unit (μm^3) in the groups.

The injectable biomaterials that are stiffer than the surrounding tissue may also shield stress and impede the proper mechanotransduction necessary for extracellular matrix remodeling during *in vivo* tissue regeneration. On the other side, very soft hydrogels may lead to excessive mechanical stress on fibroblast cells. This may stimulate the generation of smooth muscle actin (SMA) positive myofibroblasts [24] and result in scarred tissue formation. Therefore, the amount of the mechanical stress experienced by the cells can be effectively modulated by the viscoelastic properties of the injectable biomaterial [25]. The shear viscoelastic properties of the CS hydrogels (table 1) were in the range of those of human [26] and pig [27] VFLP and below those of rabbit [28] and rat [29] VFLP tissues. In particular, the storage shear moduli of human VFLP were between 10-250 Pa and 10- 100 Pa for male and female subjects, respectively. Our measurements indicate that the groups 4, 5, and 6 mimic the shear storage modulus of male VFLP, and groups 5 and 6 mimic that of female VFLP. Since groups 4, 5, and 6 were also shown to have favorable cellular viability, these groups were selected for the remainder studies, i.e., migration and degradation studies.

Table 1. Viscoelastic properties of GCs/Gy hydrogels from shear rheometry measurements.

Group number (Gy concentration)	Storage shear modulus (Pa)	Loss modulus (Pa)
#1 (0.020%)	629±35	9±1
#2 (0.015%)	560±28	9±1
#3 (0.010%)	489±41	8±1
#4 (0.005%)	307±25	4±1
#5 (0.005%)	149±31	3±1
#6 (0.0025%)	55±17	3±1

3.3. Cell migration

The cell migration speed as well as the total displacement of the cells during 4 hours imaging is shown in Fig. 3. Their storage shear modulus is in the range of that of human VFLP. The average cell speed was similar between the groups 4, 5, and 6. The ability of the cell to move freely inside the GCs/Gy hydrogel will allow cells to migrate into the injectable scaffolds from the surrounding tissue. The disability of cells to move into the matrix may result in foreign body reaction and fibrous capsule formation around the injected biomaterial [30]. These results confirm the ability of cells to move inside the Cs hydrogels. This characteristic can potentially be used to recruit patients own cells from the surrounding tissue.

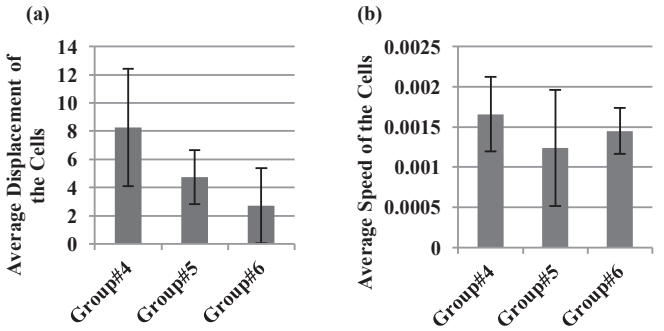

Fig. 3. Cell migration results for groups 4, 5, and 6 with Glyoxal concentrations of 0.0075%, 0.005%, 0.0025%, respectively. (a) Average displacement of the I-HVFFs in three hydrogel groups. (b) The associate average speed.

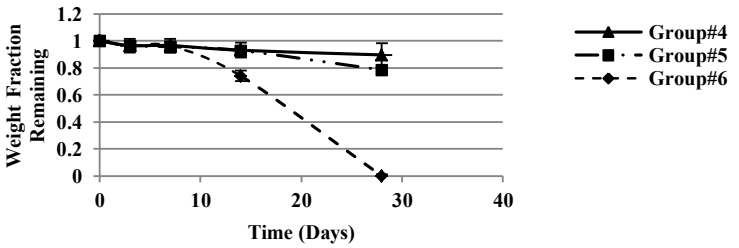

Fig. 4. In vitro enzymatic degradation for groups 4, 5, and 6 with Glyoxal concentrations of 0.0075%, 0.005%, 0.0025%, respectively.

3.4. Enzymatic degradation

The degradation profile for groups 4, 5, and 6 are shown in Fig. 4. Group 6 completely degraded in four weeks with half-life of 18 days. The degradation rate slows down significantly with the increase of cross-linker concentration. Group 4 and 5 degraded much slower compared to group 6. Following 4 weeks incubation in the enzyme solution, 10% and 21% of the hydrogel groups 4, and 5 degraded, respectively. The degradation profile should be commensurate with the formation of neo-LP tissue. However, no standard evaluation criterion exists to assess the degradation rate of the scaffolds for VFLP tissue regeneration. We estimate that a total degradation time between one to three months is required. This time period is correlated with the end of inflammation and granulation tissue formation, and varies across individuals. We speculate that one to three months residence of the scaffold material is crucial for its constant interactions with patients' cells, such as macrophages and fibroblast cells. These interactions are intended to modulate wound healing towards scar-free neo-LP tissue regeneration.

4. Conclusion

Glycol chitosan/glyoxal hydrogels with lower concentrations of Gy (≤0.0075%) were shown to support the fibroblast cells viability, and their viscoelastic properties were in the range of those of human VFLP. It was shown that by tuning the concentration of the Gy cross-linker, the degradation time of over one month was achieved. The VF fibroblasts were observed to move freely inside the GCS/Gy hydrogels. In conclusion, the GCs/Gy hydrogels are potential candidates for VF tissue regeneration.

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